Ankylosing spondylitis: an autoimmune disease?

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Abstract

Identification of several autoantibodies in serum samples from patients with ankylosing spondylitis or suspected ankylosing spondylitis is reported. Five antibodies associated with ankylosing spondylitis were identified by applying cytoimmunofluorescence and immunoblotting techniques to antigen pools from insect tissue. At least one of these antibodies was found in 82% of serum samples from patients with ankylosing spondylitis.

A 36 kD drosophila antigen, which showed the most common and most dominant reaction, was further purified and isolated. Thirty two (34%) of the serum samples from 95 patients with definite ankylosing spondylitis and 12 (28%) of the serum samples from 43 patients with suspected ankylosing spondylitis reacted with this antigen. Antibodies purified from the 36 kD antigen reacted specifically with a 69 kD antigen present in separations of total protein preparations from human lymphocytes and HeLa cells.

The 36 kD antibody was not found in 29 patients with rheumatoid arthritis nor in 38 apparently healthy controls. The prevalence of the 36 kD antibody was comparable in HLA-B27 positive and negative patients. In addition, the same immunoreaction was found in patients with so called 'seronegative' spondylarthropathies, particularly of the ankylosing spondylitis-type, suggesting that this antibody is specific for ankylosing spondylitis or other 'seronegative' spondylarthropathies with the typical clinical and radiological changes of ankylosing spondylitis.

Figure 1 Spectrum of diagnoses in the Düsseldorf database for rheumatic diseases (1999 diagnoses in 1738 patients).

larval salivary glands of *Drosophila melanogaster*, was first described.¹⁰ In 1987 four additional insect antigens were identified (36, 45, 52, and 74 kD), which reacted specifically with antibodies present in the serum of patients with ankylosing spondylitis.¹¹ Cytoimmunofluorescence and immunoblotting techniques showed that at least one of these antibodies was present in 82% of all patients with definite ankylosing spondylitis and patients with suspected disease as well.

Since 1973⁶ the strong correlation between ankylosing spondylitis and the HLA-B27 haplotype has been well recorded. No difference, however, was found between the nucleotide sequence of HLA-B27 genes from a healthy subject and from a patient with ankylosing spondylitis.¹² The structural identity of the HLA-B27 protein molecules in patients with ankylosing spondylitis and normal subjects has also been reported.¹³

The prevalence of ankylosing spondylitis was first estimated by De Blecourt in 1961⁵ to be 0.2% in men and 0.03% in women. In 1975 Calin and Fries calculated that 1.6% of the white population probably develop ankylosing spondylitis, based on the fact that the HLA-B27 haplotype is found in 8% of the white population.⁸ An improved diagnosis based on serological data would reduce this large number of apparently undiagnosed patients.

Here we report the identification of xenotypic and homotypic antigens reacting with antibodies specifically present in the serum of patients with ankylosing spondylitis, thus proving ankylosing spondylitis to be an autoimmune disease.

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Ankylosing spondylitis is the most common 'seronegative' spondylarthropathy. The low sensitivity of the New York criteria for the diagnosis of ankylosing spondylitis,^{1,2} especially in the early stages of the disease, and the lack of specific serological markers cause problems with diagnosis.³⁻⁸ Figure 1 shows a representative spectrum of rheumatic diseases reported in the *Düsseldorf Rheumaregister*, a database comprising about 2000 patients.⁹ In comparison with the other diseases ankylosing spondylitis shows the highest proportion of suspected diagnoses. Application of serological parameters specific for ankylosing spondylitis could lead to an earlier diagnosis of the disease with higher confidence and in greater number.

In 1984 an antibody specific for ankylosing spondylitis, which reacted with the 93 D heat shock puff of polythene chromosomes from

Materials and methods

SERUM SAMPLES

All serum samples were collected in the department of medicine C, endocrinology and rheumatology at the Heinrich-Heine University Düsseldorf, FRG. Diagnosis of ankylosing spondylitis and seronegative spondylarthropathies was performed according to the New York criteria^{1,2} and of rheumatoid arthritis according to the American Rheumatism Association criteria.¹⁴ All patients' serum samples were tested by counterimmunoelectrophoresis for the absence of antibodies to extractable nuclear antigens—for example, RNP, Sm, SSA, SSB. The serum samples were stored at -80°C and diluted in phosphate buffered saline before use.

CELL CULTURE

The stable KC cell line derived from *Drosophila melanogaster* embryos was cultured at 25°C and suspended in medium D-22 free from serum.^{15,16} The stable H-33 cell line from *D. hydei* embryos was cultured at 25°C in a medium supplemented with 10% fetal calf serum.^{17,18} HeLa S3 cells were cultured at 37°C suspended in a modified minimum essential medium containing L-glutamine, non-essential amino acids, fetal bovine serum, antibiotics, but no sodium bicarbonate.^{19,20}

HARVESTING OF CELLS

Lymphocytes were isolated from heparinised human venous blood provided by Professor Brüster (Institut für Blutgerinnungswesen und Transfusionsforschung, Heinrich-Heine-Universität, Düsseldorf) by differential centrifugation on a Ficoll-Paque solution ($\rho = 1.1 \text{ g/ml}$).^{21,22} After recovery from the interface they were subjected twice to a short washing step with lymphocyte culture medium RPMI. HeLa, H-33, and KC cells were harvested by centrifugation and washed twice in balanced salt solution.

GENERAL PROCEDURE FOR TOTAL PROTEIN PREPARATION

Most essential for reproducible isolation of undegraded material is the immediate lysis of the cell suspension or tissue (pulverised in liquid nitrogen) in the presence of sarcosyl, urea, and β -mercaptoethanol in a tight fitting Dounce homogeniser at $60\text{--}70^{\circ}\text{C}$. Subsequent separation from nucleic acids and polysaccharides is achieved by centrifugation in a CsCl solution with a final density of about 1.6 g/ml . The resulting float contains all peptides except for a few dense glycoproteins and can be stored below -15°C undegraded for any length of time. For good and reproducible electrophoretic separations of the isolated antigens it is necessary to remove most of the sarcosyl and CsCl. This is achieved by quick (2×30 minutes) dialysis against a buffer (containing a low concentration of the ions defining the stacking gel conditions applied in the subsequent sodium dodecylsulphate-polyacrylamide gel electro-

phoresis (SDS-PAGE)) at the lowest possible temperature in the presence of a non-ionic detergent in a flabby dialysis bag (measuring no more than 2 mm in height when lying on a flat support).

PREPARATION OF TOTAL PROTEIN FROM LYMPHOCYTES, KC, H-33, OR HeLa CELLS

The following two buffers were used: buffer A: 6 M urea, 0.1 M NaCl, 5% β -mercaptoethanol, 1% sarcosyl, 10 mM TRIS/HCl pH 7.2; buffer B: as buffer A with sarcosyl replaced by 3% Nonidet P-40. After homogenisation in buffer A the different lysates were heated to 60°C for 10 minutes. The proteins were then collected as a float after one hour's centrifugation at 30 000 rpm (Beckman SW60 rotor= $120\,000 \text{ g}$) in CsCl (final density 1.6 g/ml). The protein float was dissolved in buffer B and dialysed against TRIS/HCl buffer (25 mM, pH 7.4) and, finally, precipitated with ethanol.

IMMUNOBLOTS

The proteins were separated electrophoretically in linear pore gradient disc gels (10%–20% polyacrylamide, 2 mm thick) in the presence of urea (4 mol/l) and sodium dodecyl sulphate (0.1%).²³ The protein separations were blotted to nitrocellulose sheets (semidry²⁴); transfer results were visualised by staining with ponceau S. The sheets were then cut into strips and incubated with human serum samples diluted 1:50 in phosphate buffered saline after blocking the nitrocellulose with bovine serum albumin (0.1%) and Nonidet P-40 (1%). Immuno-reactions were visualised by a biotinylated antihuman immunoglobulin (RPN 1003; Amersham), a streptavidin-peroxidase complex (RPN 1051; Amersham), and diaminobenzidine/ H_2O_2 or a fluorescein isothiocyanate conjugated sheep antihuman immunoglobulin (MF 01, Wellcome).

PURIFICATION OF ANTIBODIES

Except for the visualisation the protein blots were treated like immunoblots. The band containing the antibody-antigen complex was cut out. The antibodies were eluted for 15 minutes with 3 M potassium thiocyanate and 0.1% bovine serum albumin in phosphate buffered saline and centrifuged in Centricon micro-concentrators (Amicon) for desalting and concentration.^{25,26} The purified antibody solution was stored at 8°C .

TWO DIMENSIONAL ELECTROPHORESIS

Proteins were focused in either gel rods or horizontal gels containing 9 M urea, 2% Nonidet P-40, and 2% ampholytes (pH 3.5–10) until the basic marker protein cytochrome c reached the cathodic end. Before separation according to molecular weight using SDS-PAGE the gels were equilibrated in a buffer containing 10% glycerol, 2% sodium dodecyl sulphate, 5% β -mercaptoethanol, and 60 mM TRIS/HCl, pH 6.8, for one hour.^{27–30}

Results

In immunoblotting experiments we found a strong immune response with the 36 kD drosophila protein in 32 (34%) serum samples from patients with definite ankylosing spondylitis and in 12 (28%) samples from patients with suspected ankylosing spondylitis (table, fig 2). Immunoreactions of this type were identical in protein preparations from serum free cultured KC cells and from fetal calf serum supplemented H-33 cells.

To prove the specificity of the antibody to the 36 kD antigen we tested several serum samples from patients with other 'seronegative' spondylarthropathies—for example, psoriatic arthritis, Crohn's disease—and other systemic rheumatic diseases—for example, rheumatoid arthritis. As shown in the table 15 (44%) of 34 serum samples from patients with 'seronegative' spondylarthropathies with diagnosed ankylosing spondylitis and only two (13%) of 15 samples from patients with other 'seronegative' arthropathies not of the ankylosing spondylitis type reacted with the 36 kD antigen, whereas

Figure 2 Staining of a 36 kD KC cell protein specific for ankylosing spondylitis. Western blot of a separation of a total protein preparation from the *Drosophila melanogaster* embryonic KC cell line incubated with serum from 13 patients with ankylosing spondylitis (lanes 3–15) and pooled normal control serum (lane 2). The reactions were visualised by a biotinylated secondary antibody, a streptavidin-peroxidase complex, and diaminobenzidine. Serum samples from the patients show a specific reaction with a 36 kD protein (arrow). Lane 1, the control, shows the reaction of the secondary antibody alone. The faint bands at 36 kD visible in lanes 1 and 2 result from a cross reaction with the secondary antibody. These as well as most of the other bands cannot be seen in reactions with a fluorescein isothiocyanate conjugated sheep antihuman immunoglobulin antibody (MF 01 Wellcome) as shown in ref 11.

Prevalence of antibodies to the *Drosophila melanogaster* 36 kD antigen in serum samples from patients with various rheumatic diseases

Diagnoses	Total number of tested serum samples	No (%) showing positive reaction with the 36 kD antigen
Definite AS *	95	32 (34)
Suspected AS	43	12 (28)
Definite and suspected AS		
HLA-B27 positive	120	42 (35)
HLA-B27 negative	18	5 (28)
Seronegative spondylarthropathies with diagnosed AS	34	15 (44)
Crohn's disease	23	10 (43)
Psoriatic arthritis	11	5 (45)
Seronegative spondylarthropathies without diagnosed AS	15	2 (13)
Rheumatoid arthritis	29	0
Healthy controls	38	0

*AS=ankylosing spondylitis.

serum samples both from patients with rheumatoid arthritis and from apparently healthy controls did not show a positive reaction.

To test for a possible correlation between the presence of the antibody to the 36 kD antigen and the expression of HLA-B27 we screened HLA-B27 positive and negative serum samples. In the patients with definite and suspected ankylosing spondylitis positive immune reactions with the 36 kD antigen were found in 42 (35%) of 120 HLA-B27 positive serum samples and in five out of 18 (28%) HLA-B27 negative samples (table).

The presence of antibodies to the 36 kD antigen in patients with ankylosing spondylitis seems to be independent of the patients' age, sex, and disease activity as indicated by erythrocyte sedimentation rate and radiological changes.

For further characterisation of the 36 kD antigen the total KC protein was separated in two dimensional gels with non-equilibrium, pH gradient electrophoresis as first dimension and SDS-PAGE as second. This particular gel was blotted one after the other to three different nitrocellulose sheets. The first and third blot showed a single spot at 36 kD and a basic pH reacting strongly with pooled ankylosing spondylitis serum samples, whereas the second blot did not show any reaction at all when incubated with a normal serum pool, thus proving the selective and highly specific reaction of the ankylosing spondylitis specific antibody (fig 3). Antibodies eluted from these spots in turn specifically reacted with the 36 kD band in Western blots of total protein preparations of KC cells (data not shown). The 36 kD protein showed an isoelectric point of 9.0 when focused under equilibrium conditions. When the serum of patients with ankylosing spondylitis and antibodies purified from the 36 kD drosophila protein were applied to Western blots of human lymphocyte and HeLa proteins, a 69 kD human antigen could be identified (figs 4A and B). The eluted antibody was reincubated with a Western

Figure 3 Reaction of the 36 kD *Drosophila* protein selective and specific for ankylosing spondylitis. Two dimensional separation of total protein preparation of KC cells: non-equilibrium, pH gradient electrophoresis (NEPHGE) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was blotted to three different nitrocellulose filters (A, B, C) for 20 minutes each. Blots A and C were reacted with pooled ankylosing spondylitis serum samples, whereas blot B was incubated with a pool of normal serum samples as control. Detection of the bound human antibodies occurred with a fluorescein isothiocyanate conjugated sheep antihuman immunoglobulin secondary antibody. Blots A and C show a strong signal at 36 kD (arrows), whereas blot B shows no signal.

Figure 4 Reaction with (A) a human lymphocyte protein and (B) a HeLa protein specific for ankylosing spondylitis. Immunoblots of (A) lymphocyte proteins and (B) HeLa proteins were incubated with pooled ankylosing spondylitis serum samples (lane 3), pooled normal control serum samples (lane 2), and antibodies eluted from the 36 kD *Drosophila melanogaster* protein after reaction with pooled ankylosing spondylitis serum samples (lane 4). The reactions were visualised by a biotinylated secondary antibody, a streptavidin-peroxidase complex, and diaminobenzidine. The pooled ankylosing spondylitis serum samples and the purified antibodies to the 36 kD antigen show specific reactions with a 69 kD lymphocyte and a 69 kD HeLa protein (arrows). Other bands visible on lane 4 are reactions of the secondary antibody. Lane 1, the control, shows the reaction of the secondary antibody alone.

blot identical to the one shown in fig 2. A single band of 36 kD reacted, proving the quality and specificity of the eluted antibody (fig 5). Elution of the antibodies reacting with the 69 kD human lymphocyte antigen and reincubation on KC protein immunoblots showed a specific reaction with a 36 kD drosophila antigen (data not shown).

Discussion

The specificity and prevalence of the antibodies to the 36 kD antigen, detectable in immunoblots of *D melanogaster* and *D hydei* protein preparations, as well as those reacting with the 93 D heat shock puff substantially improve the chances of diagnosing ankylosing spondylitis and thus help to differentiate it from other systemic rheumatic diseases.

Introduction of these serological markers of ankylosing spondylitis aids the diagnostic process in the early stages and in women, who very often have a milder form of disease. Furthermore, the antibody to the 36 kD antigen supports the identification of other 'sero-negative' spondylarthropathies, which are characterised by symptoms of ankylosing spondylitis. According to these results, the 36 kD antibody could be used to identify patients with ankylosing spondylitis or related diseases, like psoriatic arthritis or inflammatory bowel disease.

Figure 5 Quality of the eluted 36 kD specific antibody. The immunoblot of *Drosophila melanogaster* proteins derived from the embryonic KC cell line was incubated with antibodies purified from the reaction of ankylosing spondylitis serum samples with the 36 kD protein (lane 1), a strongly reacting serum from a patient with ankylosing spondylitis (lane 2), a pool of ankylosing spondylitis serum samples (lane 3), and pooled normal control serum samples (lane 4). The reactions were visualised by a biotinylated secondary antibody, a streptavidin-peroxidase complex and diaminobenzidine. The serum samples from the patients with ankylosing spondylitis and the purified antibodies to the 36 kD antigen show a specific reaction with the 36 kD protein (arrow). Lane 5, the control, shows the reaction of the secondary antibody alone (lane 5).

Ankylosing spondylitis and other 'sero-negative' spondylarthropathies are most tightly linked to the major histocompatibility complex (MHC) class I locus, the HLA-B27 haplotype.³¹⁻³² The molecule's function as a class I restriction element for cytotoxic lymphocytes is not altered by molecular variations of HLA-B27: the diseases are associated with different variants of HLA-B27.

The 36 kD antibodies are not linked to HLA-B27, as can be judged from the fact that HLA-B27 negative ankylosing spondylitis serum samples also react with the 36 kD antigen. It has been suggested, however, that the basis of HLA-B27 disease association might be a non-functional contribution of an HLA-B27 sequence or a closely linked gene.³³⁻³⁵ Using total protein preparations from human tissue, we were able to show for the first time that antibodies eluted from the 36 kD drosophila antigen cross react on immunoblots with lymphocyte and HeLa proteins. This means that the 36 kD antibody is an autoantibody and consequently, we feel ankylosing spondylitis should correctly be

classified as an autoimmune disease, though this suggestion is still a matter for debate.³⁶⁻³⁹

As HLA disease associations are not a mere genetic coincidence, but rather a common feature of many immunologically mediated disease processes, it is still possible that HLA-B27 in patients developing ankylosing spondylitis undergoes structural modification owing to the action of exterior agents (viruses, bacteria, etc). Cause of the disease may be multifactorial—HLA-B27 interacting with other factors, both genetic and environmental (low concordance rates for disease in monozygotic twins).⁴⁰⁻⁴²

The cause of ankylosing spondylitis is not necessarily an autoantibody, but might also be a malfunction of cellular immunity. We consider one possibility might be an immunoreactive lymphocyte clone producing the antibody to the 36 kD antigen, which will be found only in patients with ankylosing spondylitis. According to this theory it is important that the 36 kD antibody shows strong immunostaining with lymphocyte proteins. In this context we would like to point out that bacterially induced release of cytokines from T cells, especially interferon, can increase the expression of MHC coding class I and II structures in such a way that cells normally unable to present an antigen are now able to do so. A higher rate of foreign antigen presentation leads to local activation and maturation of T and B cells, and also of cytotoxic cells, which are non-MHC restricted. Normal cell surface structures in the neighbourhood of an MHC structure may now become immunogenic and cause autoaggressive reactions against normal cell antigens.⁴³

The structure and function of the human antigens recognised by the antibodies specific for ankylosing spondylitis are still unknown. Preliminary results^{10 11 44} show that the drosophila antigens are neither degradation products of a larger entity nor heat shock proteins or members of the well known RNP—for example, snRNP, proteins. Furthermore, the autoantibodies described here are definitely different from those reported by Schwimmbeck *et al*,⁴⁵ which recognised the HLA-B27 antigens with a molecular weight of 45 kD and 12 kD.

A prerequisite for the isolation of affinity purified monospecific 36 kD antibodies is purification of the 36 kD antigen by two dimensional gel electrophoresis. The monospecific antibodies are used for screening human expression vector libraries to obtain the respective human antigen as fusion protein. Analysis of the gene coding for this antigen will open the way for investigation of the pathogenesis and cause of ankylosing spondylitis and other 'seronegative' spondylarthropathies with symptoms of ankylosing spondylitis.

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